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Antisense RNA that affects *Rhodopseudomonas palustris* quorum-sensing signal receptor expression

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Quorum sensing in the bacterium *Rhodopseudomonas palustris* involves the RpaI signal synthase, which produces *p*-coumaroyl-homoserine lactone (*p*C-HSL) and RpaR, which is a *p*C-HSL–dependent transcriptional activator. There is also an antisense *rpaR* transcript (as*rpaR*) of unknown function. Recent RNAseq studies have revealed that bacterial antisense RNAs are abundant, but little is known about the function of these molecules. Because as*rpaR* expression is quorum sensing dependent, we sought to characterize its production and function. We show that as*rpaR* is approximately 300–600 bases and is produced in response to *p*C-HSL and RpaR. There is an RpaR-binding site centered 51.5 bp from the mapped as*rpaR* transcript start site. We show that as*rpaR* overexpression reduces RpaR levels, *rpaI* expression, and *p*C-HSL production. We also generated an as*rpaR* mutant, which shows elevated RpaR levels, and elevated *rpaI* expression. Thus, as*rpaR* inhibits *rpaR* translation, and this inhibition results in suppression of RpaR-dependent *rpaI* expression and, thus, *p*C-HSL production. The *R. palustris* as*rpaR* represents an antisense RNA for which an activity can be measured and for which a distinct regulatory circuit related to a function is elucidated. It also represents yet another subtle regulatory layer for acyl-homoserine lactone quorum-sensing signal-responsive transcription factors.

bacterial communication | translational control

We have been interested in the *Rhodopseudomonas palustris* quorum-sensing system because although many *Proteobacteria* use acyl-homoserine lactone (AHL) quorum sensing signals, *R. palustris* is unusual in that it uses an aryl-HSL, *p*-coumaroyl-HSL (*p*C-HSL) rather than a fatty AHL. The *R. palustris* quorum sensing circuit is also unusual in that *p*C-HSL is not made entirely from endogenously produced substrates. Rather, its synthesis requires exogenous *p*-coumarate (1). The *R. palustris* signal synthesis gene, *rpaI*, codes for a member of the large LuxI family of AHL synthases, and the adjacent signal receptor gene, *rpaR*, codes for a member of the LuxR family of transcription factors. Although *rpaI* and *rpaR* are not cotranscribed, they are adjacent and show the same orientation (Fig. 1*A*). The *rpaR* translational stop codon is 86 bp upstream of the *rpaI* transcriptional start site. The *rpaI* gene is positively autoregulated by its product *p*C-HSL and RpaR, which binds to an inverted repeat element called the RpaR-box centered 48.5 bp from the *rpaI* transcript start site (2).

We recently performed an RNAseq analysis to identify quorum-sensing-dependent transcripts in *R. palustris* and discovered that there is an *rpaR* antisense RNA (asRNA) produced in response to *p*C-HSL and RpaR. This asRNA, which we call antisense *rpaR* transcript (as*rpaR*), is among the transcripts showing the greatest *p*C-HSL dependence (2). We have pursued an investigation of as*rpaR* for the following reasons: There are extensive regulatory layers on LuxR homologs in different bacteria (3–9), and the implication is that precise fine-tuning of LuxR levels is a critical element of quorum sensing. The *p*C-HSL-RpaR dependence of as*rpaR* transcription leads us to speculate that because this RNA is complementary to *rpaR* message, it could exert a regulatory effect on quorum sensing. Regulation by an antisense RNA would represent yet another element that can fine-tune quorum sensing signal receptor activity. Furthermore, next-generation sequencing technologies have revealed that asRNAs are quite prevalent in bacteria. Although there is an extensive literature on regulatory functions of *trans*-encoded small-regulatory RNAs in bacteria, relatively little is known about *cis*-encoded asRNAs (10, 11). There has been speculation that the preponderance of asRNAs detected by next-generation sequencing by and large represents nothing more than insignificant mispriming events (11). There are, however, a few examples of bona fide asRNAs with known regulatory activities (12, 13).

Results

Characterization of as*rpaR*. Our published RNAseq analysis of *R. palustris* quorum-sensing-controlled genes revealed a *p*C-HSL-activated antisense *rpaR* transcript or transcripts that appeared to overlap most of the length of the *rpaR* mRNA. The results indicated there was an asRNA or asRNAs extending from somewhere around 50 bases upstream of the *rpaR* stop codon to 604 bases upstream of the stop codon (2). A unique feature of the *R. palustris* *p*C-HSL quorum sensing system is that the signal is produced only if *p*-coumarate is added to the growth medium. Thus, the *p*C-HSL-dependent transcriptome can be identified by comparing wild-type cells grown with either *p*-coumarate or the *p*C-HSL signal itself to cells grown without either of these molecules (1, 2). Expression of *rpaR* is not affected by *p*C-HSL but the antisense *rpaR*, which we term as*rpaR*, shows >20-fold *p*C-HSL induction (2). To determine whether *p*C-HSL induced synthesis of a series of small *rpaR* antisense molecules or whether longer antisense molecules were produced, we performed a Northern blot analysis with RNA harvested from wild-type and *rpaR*-mutant *R. palustris* cells by using strand-specific RNA probes corresponding to 207 bp of the 3′ end of *rpaR* (*Materials and Methods*). We detected asRNA between 300 and approximately 600 bases from cells grown with *p*C-HSL (Fig. 1*B*). As expected, we detected transcripts (400–800 bases) when probing for *rpaR* mRNA from wild-type cells grown with or without *p*C-HSL. As a control, we show that there is no detectable *rpaR* message in the *rpaR* deletion mutant CGA850 (2) (Fig. 1*B*).

We mapped the as*rpaR* transcript start site to a guanine positioned 41 bases from the *rpaR* stop codon by primer extension and S1 nuclease protection analyses with RNA isolated from cells grown with or without *p*-coumarate, or RNA isolated from the *rpaR* deletion mutant (Fig. 2 *A* and *B* and Fig. S1). We used a 3′-RACE analysis to detect as*rpaR* 3′ ends. We sequenced 11

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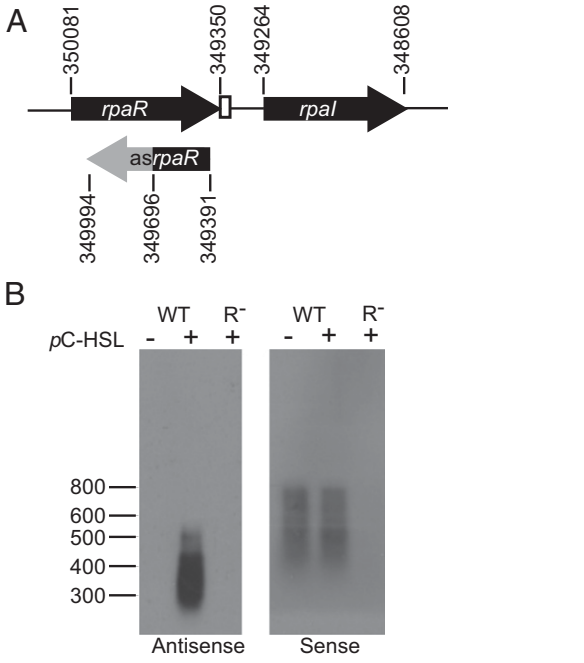

Fig. 1. The *R. palustris rpaR-rpaI* region and expression of as*rpaR* and *rpaR* mRNA. (*A*) Map of the *rpaR-rpaI* region. The intergenic region is 86 bp and contains an inverted repeat RpaR-binding site (white box) (2). Numbers refer to nucleotide coordinates in the *R. palustris* CGA009 genome (32). The as*rpaR* transcript is depicted as a two-toned arrow. The black shading indicates the shortest product detected (304 bases), and the gray shading indicates the longest product detected (604 bases) (Table S1). (*B*) Northern-blot analysis of RNA from the wild-type CGA009 (WT) and the *rpaR*-deletion mutant CGA850 (R⁻) grown in the presence and absence of 250 nM *p*C-HSL. Blots were probed for products specific to as*rpaR* (antisense) or *rpaR* mRNA (sense) as described in *Materials and Methods*. The locations of size markers (bases) are indicated on the left.

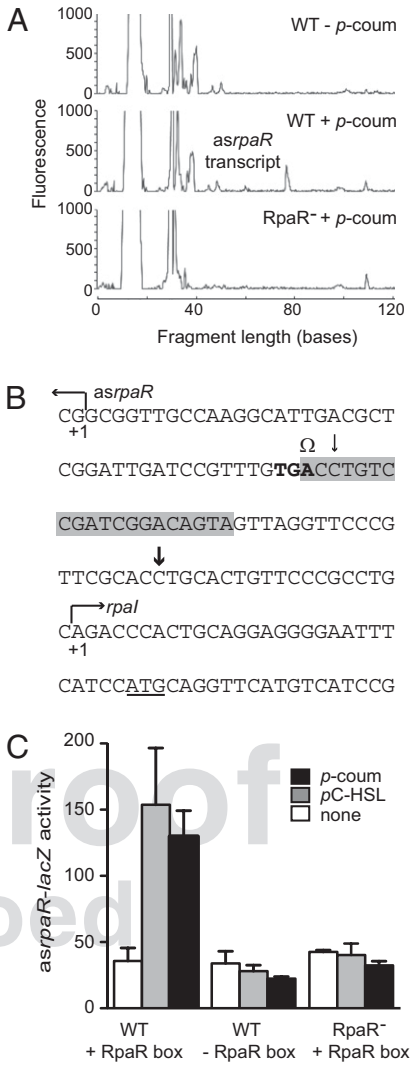

Fig. 2. The as*rpaR* transcript start site and the dependence of as*rpaR* expression on the intergenic RpaR box. (*A*) Primer extension analysis of as*rpaR* from the *R. palustris* wild type (CGA009) grown with or without *p*-coumarate and the RpaR mutant (CGA850) grown with *p*-coumarate. (*B*) Sequence of the *rpaR-rpaI* intergenic region. Transcription start sites for as*rpaR* (coordinate 349392) and *rpaI* (coordinate 349293) are indicated by +1 arrows. The RpaR-binding site (RpaR box) is indicated by the gray box (coordinates 349350–349331), the *rpaI* start codon (coordinates 349264–349262) is underlined, and the *rpaR* stop codon is bolded (coordinates 349352–349350). Arrows indicate the 5′-most position of the as*rpaR-lacZ* fusions for pHH102 (thick arrow) and pHH103 (thin arrow). The Ω designates the location of the Ω-cassette insertion in strain CGA854 as described in *Materials and Methods*. (*C*) Transcription of as*rpaR* requires both RpaR and the RpaR-binding site. Wild type (WT) or the RpaR⁻ mutant containing pHH102 (intact RpaR-box) or pHH103 (RpaR-box deletion) was assessed for as*rpaR-lacZ* activity. Cells were grown with the following additions: none (white bars), 250 nM *p*C-HSL (gray bars), or 0.5 mM *p*-coumarate (black bars). Data are the means of three biological replicates, and the error bars indicate ranges.

different clones and found as*rpaR* sizes to vary from 305 to 468 bp (Table S1), in agreement with the sizes of the most abundant species detected by Northern analysis (Fig. 1*B*). It is not clear whether there may be several different transcripts synthesized or whether the size range results from some degradation of a single long element, but it is clear that *p*C-HSL induces asRNAs of considerable length. We also observed a range in the sizes of *rpaR* mRNA. The *rpaR* ORF is 732 bp, corresponding well with the upper end of *rpaR* transcript sizes observed in the Northern blot analysis (Fig. 1 *B*).

The as*rpaR* transcript start site is located 51.5 bp from the RpaR box required for *p*C-HSL induction of *rpaI* (Fig. 2 *A* and *B*). Because this spacing is similar to that between the *rpaI* transcript start and the RpaR box (48.5 bp), we reasoned that the RpaR box might be involved in *p*C-HSL induction of as*rpaR*. To test this idea, we constructed two as*rpaR*-promoter-*lacZ* plasmids: One contained the as*rpaR* transcriptional start fused to *lacZ* and extended 81 bp upstream of the as*rpaR* start site (pHH102; Fig. 2*B*, thick arrow), and the other extended 44 bp from the as*rpaR* transcript start and, thus, contained only 3 bp of the RpaR-box (pHH103; Fig. 2*B*, thin arrow). Expression of *lacZ* in *R. palustris* containing the complete RpaR-box plasmid was induced by *p*C-HSL, but *p*C-HSL did not induce *lacZ* in *R. palustris* carrying the RpaR-box-deletion construct (Fig. 2*C*). As a control, we used the *rpaR* deletion mutant to show that *p*C-HSL induction of *lacZ* in cells containing the complete RpaR-box-as*rpaR-lacZ* construct required RpaR (Fig. 2*C*). Thus, we conclude that induction of both *rpaI* and as*rpaR* depends on a single RpaR box in the *rpaR-rpaI* intergenic region and also depends on RpaR and *p*C-HSL. It should be pointed out here that a number of genes are activated by RpaR and *p*C-HSL, but electromobility shift assays show that among these genes, only the *rpaI* operon is a direct target of RpaR (2). By using the *rpaI* RpaR-binding site, as*rpaR* is also directly regulated by RpaR.

Evidence That as*rpaR* Is Not Translated. Although uncommon, there is precedence for overlapping (or cryptic) protein-encoding antisense genes in bacteria (14). We examined the as*rpaR* DNA

sequence for potential ORFs by using ORFfinder (www.ncbi.nlm.nih.gov/gorf/gorf.html) and identified a single ORF encoding a polypeptide >50 aa residues (480 bp in length). We tested the idea that as*rpaR* codes for a polypeptide in several ways, and all of the evidence is consistent with the conclusion that it does not. First, a BLAST analysis showed these peptides do not match any known sequences. Second, we have a large database of peptide fragments from *R. palustris* grown in the presence of *p*-coumarate (15), and we did not find peptides corresponding to the ORF. Third, we created an as*rpaR-lacZ* translational fusion plasmid. β-Galactosidase activity in wild-type *R. palustris* containing this plasmid was negligible and unaffected by growth in the presence of *p*-coumarate. Fourth, we constructed an as*rpaR* plasmid containing a hexaHis-tag–coding sequence at the 3′ end of the predicted 480-bp ORF. We could not detect the putative His-tagged protein by Western blotting with anti-His tag antiserum. Fifth, and most convincing, we constructed an *R. palustris* as*rpaR* mutant (CGA854), with a stop codon substituted for the glutamate-codon at position 11 in the ORF. This substitution was designed so that it did not alter the RpaR protein sequence. In experiments described below, we show measurable activity of as*rpaR*, and the activity is not affected in the stop codon substitution mutant strain.

Overexpression of as*rpaR* Reduces RpaR Polypeptide Levels, *rpaI* Transcription, and *p*C-HSL Production. Because the RNAseq (2) and Northern blot analyses (Fig. 1*B*) indicated that *p*C-HSL did not influence *rpaR* mRNA levels, but activated as*rpaR* expression, we asked whether as*rpaR* might interfere with *rpaR* translation. Levels of RpaR in *R. palustris* are low, and for technical reasons, we could not detect native RpaR or even a hexaHis-RpaR encoded on an expression plasmid by Western blotting. We thus sought evidence that as*rpaR* overexpression would affect quorum sensing in *R. palustris*. We predicted that as*rpaR* overexpression should decrease RpaR from its already low level and, thus, have a measurable affect on *rpaI* expression, which depends on RpaR and on the enzymatic product of RpaI, *p*C-HSL. Our prediction was borne out by experiments with *R. palustris* containing a glyceraldehyde-3-phosphate dehydrogenase promoter-controlled as*rpaR* construct (Fig. 3 *A* and *B*). Furthermore, as discussed above, we also used a vector expressing as*rpaR* with a stop codon substituted for the glutamate-codon at position 11 in the potential polypeptide coding sequence of as*rpaR*, and cells with this vector had a phenotype identical to that of cells with the wild-type as*rpaR* vector (Fig. 3*A*). This finding supports our view that as*rpaR* function is at the level of RNA interactions and as*rpaR* does not code for a functional protein. The data suggest as*rpaR* base pairs with *rpaR* mRNA, and basepairing reduces *rpaR* translation.

To gain more direct evidence that as*rpaR* interfered with RpaR expression, we investigated whether overproduction of as*rpaR* affected the level of a His-tagged RpaR expressed from a low-copy number plasmid in recombinant *Pseudomonas aeruginosa*, which has a high genomic GC content similar to that of *R. palustris*. For overexpression of as*rpaR*, we used a plasmid with an iso-propylthio-β-D-galactopyranoside-inducible as*rpaR* (pHH100). We assessed the level of hexaHis-RpaR by Western blotting with anti–poly-His serum. The hexaHis-RpaR was easily detected in cells that were not overexpressing as*rpaR*, whereas overexpression of as*rpaR* reduced the level of hexaHis-RpaR to below detection (Fig. 3*C*).

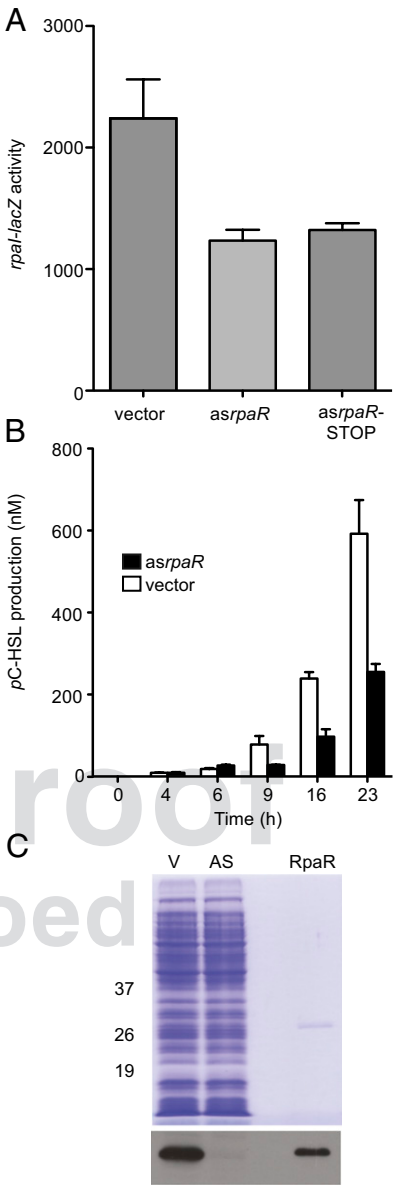

Fig. 3. Overexpression of as*rpaR* decreases RpaR levels and RpaR-dependent expression of *rpaI*. (*A*) Overexpression of as*rpaR* reduces *rpaI-lacZ* expression. β-Galactosidase activity in *R. palustris* CGA814 with the as*rpaR* expression vector pHH104 (as*rpaR*), the as*rpaR* STOP mutation vector pHH105 (as*rpaR*-STOP), or empty vector pBBPgdh (vector) grown for 16 h with 0.5 nM *p*C-HSL. (*B*) *p*C-HSL production by *R. palustris* CGA009 with pHH104 (filled bars; as*rpaR*) or pBBPgdh vector control (open bars; vector). Cells were grown in the presence of 0.5 mM *p*-coumarate. (*C*) Overexpression of as*rpaR* reduces hexaHisRpaR in recombinant *P. aeruginosa*. (*C Upper*) SDS/PAGE of soluble polypeptides from *P. aeruginosa* containing a hexaHis-RpaR expression plasmid (pHH101) plus either the as*rpaR*-expression vector pHH100 (AS) or the empty vector pQF5016b (V). Purified hexaHis-RpaR (predicted molecular mass 27,800) is included for comparison. Locations of molecular mass standards (in kilodaltons) shown on the left. (*C Lower*) HexaHis-RpaR was visualized by probing with anti–poly-His antibody. For *A* and *B*, data are the means of two biological replicates, and the error bars indicate ranges.

Reducing as*rpaR* Expression Increases RpaR Polypeptide Levels and *rpaI* Promoter Activity in *R. palustris*. Construction of a specific as*rpaR* mutant is not straightforward: Deletion of the antisense-coding DNA also disrupts the corresponding *rpaR* gene (Fig. 1*A*), and deletion of the RpaR-binding site required for as*rpaR* expression (Fig. 2*C*) reduces *rpaI* expression (2). Therefore, to create the *R. palustris* as*rpaR* mutant CGA851, we inserted an Ω stem-loop terminator fragment (16) between the as*rpaR* transcript start site and the RpaR-box (Fig. 2*B* and *Materials and Methods*). Disruption of the R-protein binding site spacing in other AHL quorum sensing systems results in loss of transcriptional activation (17). As expected, we did not detect as*rpaR* RNA in the Ω insertion mutant (Fig. 4*A*). However, the as*rpaR*-

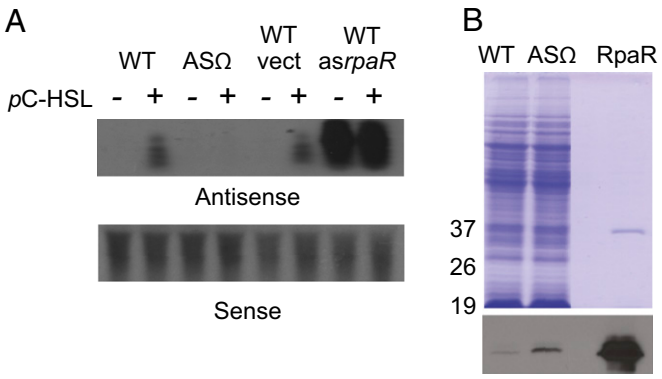

Fig. 4. Reduced as*rpaR* correlates with increased RpaR levels but not *rpaR* mRNA. (*A*) as*rpaR* (*Upper*) and *rpaR* mRNA (*Lower*) from wild type (WT), as*rpaR*-Ω (ASΩ) mutant, wild type containing pBBPgdh vector control (WT vect), and wild type containing the as*rpaR* expression vector pHH104 (WT as*rpaR*) grown in the presence and absence of *p*C-HSL. (*B*) An *R. palustris* as*rpaR* mutant has increased VSVG-RpaR levels. (*B Upper*) SDS/PAGE of soluble polypeptides from the wild type or as*rpaR*-Ω mutant grown in the presence of 250 nM *p*C-HSL. Purified VSVG-RpaR (predicted molecular mass 28,200) is included (rightmost lane) for comparison. Locations of molecular mass standards (in kilodaltons) are indicated on the left. (*B Lower*) VSVG-tagged RpaR was visualized by Western blotting with VSVG antibodies.

Ω mutant had normal levels of *rpaR* mRNA (Fig. 4*A*), suggesting the as*rpaR* does not affect *rpaR* transcription or mRNA stability.

We hypothesized the as*rpaR*-Ω mutation would affect RpaR polypeptide levels and activity. Because we were unable to detect RpaR in *R. palustris* by Western blotting with RpaR antiserum, we replaced the native *rpaR* gene with a gene coding for RpaR with a N-terminal vesicular stomatis virus glycoprotein (VSVG) epitope (*Materials and Methods*) in the wild type and the as*rpaR*-Ω mutant. We then detected VSVG-RpaR in *R. palustris* cell lysates by Western blotting with anti-VSVG serum (Fig. 4*B*). As predicted, there was more VSVG-RpaR in the as*rpaR*-Ω mutant than in the wild type (Fig. 4*B*). By using the *rpaI-lacZ* reporter we also show that the *rpaI* promoter is more active in the as*rpaR*-Ω strain than its parent (Fig. 5*A*). These data are consistent with the idea that RpaR polypeptide levels are increased when there is no as*rpaR* present to interfere with translation. We also found that *rpaI-lacZ* levels were indistinguishable between wild type and CGA854 (Fig. 5*A*), which harbors an as*rpaR* gene with a stop codon in the potential coding sequence. As discussed previously, this finding supports our view that as*rpaR* is not translated.

We generated an anti-as*rpaR* expression vector (pHH106) plasmid coding for 204 bases near the 3′ end of the *rpaR* sense mRNA (the full-length *rpaR* ORF is 732 bases). As predicted, *R. palustris* containing the anti-as*rpaR* expression vector showed a modest increase in *rpaI-lacZ* activity (Fig. 5*A*) and *p*C-HSL levels (Fig. 5*B*).

Discussion

Bacterial regulatory RNAs are important in gene control. Most studies of these regulatory RNAs have focused on *trans*-encoded small RNAs (sRNAs) known to control a range of functions. The list of sRNA-controlled functions includes examples of AHL signal receptors (18, 19). With the advent of RNAseq methods, there have been reports showing *cis*-encoded asRNAs can be quite common in bacteria (10, 11). With a few exceptions, the functional importance of these asRNAs is not understood (10, 11), and one interpretation of the RNAseq results is that in many cases, the asRNAs might have little or no biological significance. In a previously published study of quorum sensing in *R. palustris*, we discovered antisense *rpaR* RNA and an RNAseq analysis indicated its synthesis was activated by RpaR and *p*C-HSL, the quorum-sensing signal to which RpaR responds (2). The fact that the synthesis of this asRNA was regulated suggested that it might be of biological significance. We suspected that an as*rpaR* transcript might regulate some feature of quorum sensing or it might code for a regulator of quorum sensing. Thus, we chose to study it in more detail.

The *rpaR* gene is upstream of *rpaI*, and both genes are transcribed in the same direction. There are 86 bp between the *rpaR* translational stop codon and the *rpaI* transcription start site. There is an RpaR-binding site situated in the *rpaR-rpaI* intergenic region that is required for activation of *rpaI* by *p*C-HSL-bound RpaR (Fig. 1*A*). Our evidence indicates that as*rpaR* is approximately 300–600 bases in length with a transcript start site located 41 bp into the *rpaR* ORF and confirms that *p*C-HSL and RpaR activate expression of as*rpaR*, and this activation depends on the RpaR-binding site required for *rpaI* induction by *p*C-HSL and RpaR (Fig. 2). We present several lines of evidence indicating that as*rpaR* is not translated and that as*rpaR* functions as a regulatory RNA (Figs. 2–5).

Our studies of as*rpaR* overexpression strains and as*rpaR* mutants indicate that as*rpaR* represses RpaR levels, but not *rpaR* transcription, and it affects *rpaI* expression and timing of *p*C-HSL production under our experimental conditions. Thus, as*rpaR* has a measurable biological activity. We propose it serves as

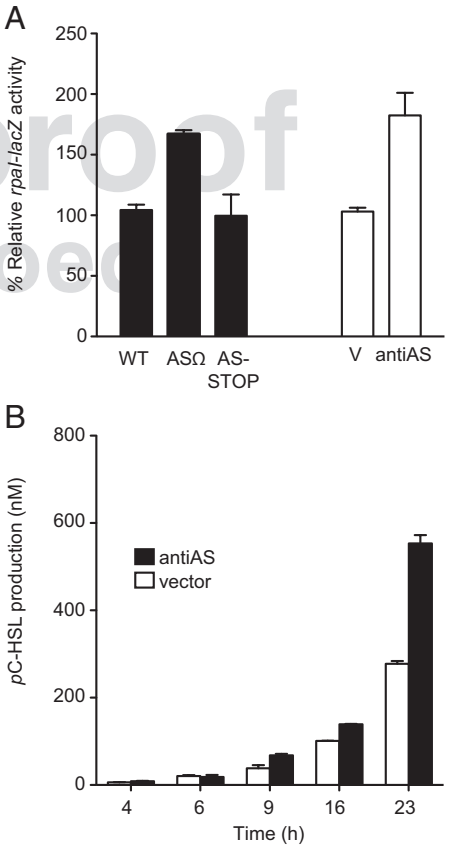

Fig. 5. Reduced as*rpaR* correlates with increased *rpaI* expression and *p*C-HSL production. (*A*) β-Galactosidase levels of *R. palustris* containing an *rpaI-lacZ* plasmid (pPrpaI) and grown with *p*C-HSL. Shown are the wild type CGA009 (WT), the as*rpaR*-Ω mutant CGA851 (ASΩ), the as*rpaR*-STOP mutant CGA853 (AS-STOP), WT with the vector control for the anti-as*rpaR,* pBBPgdh (V), or WT with the anti-as*rpaR* expression vector pHH106 (antiAS). Data for the WT, ASΩ, and AS-STOP are reported as a percent of WT and for the WT carrying the antiAS plasmid data are reported as a percent of the vector control. (*B*) *p*C-HSL production by *R. palustris* CGA009 strains carrying the anti-as*rpaR* expression construct pHH106 (filled bars; antiAS) or the vector alone (open bars; vector). Cells were grown with 0.5 mM *p*-coumarate. Data are the means of two biological replicates, and the error bars indicate ranges.

a mechanism to fine-tune cellular levels of RpaR in populations that have achieved a quorum. This proposal is supported by our finding that *rpaR* mRNA levels are not affected by induction of as*rpaR* or by inactivating mutations of as*rpaR* (Figs. 1*B* and 4*A*). We suppose the regulatory mechanism involves as*rpaR* base-pairing with *rpaR* message. Other asRNAs exert activity either by modifying levels of target transcripts or by interfering with target translation (10, 11). It does not appear that as*rpaR* affects *rpaR* mRNA levels, either via transcription interference, attenuation, or altering RNase susceptibility, because *rpaR* sense transcript levels are indistiguishable in wild-type and as*rpaR*-mutant strains (Fig. 4*A*). In fact, as*rpaR* overexpression does not affect *rpaR* mRNA levels (Fig. 4*A*) although RpaR activity is reduced (Fig. 3).

Many regulatory RNAs, including the handful of characterized asRNAs, affect translation by base pairing with the target mRNA ribosome-binding site (rbs) and interfering with ribosome binding (10, 11). This situation is unlikely to be the case for as*rpaR*, which is predicted to base pair with the 3′ region of *rpaR* mRNA. We do not know how as*rpaR* interferes with translation. There are examples of regulatory RNAs that bind their targets up- or downstream of the rbs, yet still affect translation (20–22), and it has been suggested that extended stretches of sense-asRNA duplexes might be sufficiently stable to interfere with translation (10).

In AHL quorum-sensing systems, the cellular concentration of the LuxR homolog affects the sensitivity of the system to the signal (23–25). A variety of regulatory mechanisms maintain these transcription factors at specific levels. Active *Agrobacterium tumefaciens* TraR levels are influenced posttranslationally by TrlR, a TraR homolog that does not possess a DNA binding domain. TrlR forms inactive heterodimers with full-length TraR (3). The *A. tumefaciens* quorum-sensing circuit also includes TraM an antiactivator protein that interacts with TraR to prevent its DNA binding (5, 8). *A. tumefaciens* TraM mutants respond to much lower levels of the quorum-sensing signal than does the parent (26). Antiactivator proteins have been identified in *P. aeruginosa.* The QteE (7) and QslA (6) proteins function to reduce levels of active LasR and decrease sensitivity to the quorum-sensing signal. Here, we show an additional form of quorum-sensing transcription factor control in *R. palustris*. The *R. palustris* quorum-sensing circuit can use as*rpaR* to fine-tune RpaR levels. Compared with control of a quorum-sensing signal receptor by polypeptides, control of RpaR pools by an asRNA might increase target specificity and, obviously, there could be energy efficiency because there is no translation. It has also been suggested that asRNA regulation might have an advantage over transcription regulator proteins when fast responses are useful (27, 28).

It is possible that an asRNA fine-tunes other quorum-sensing systems in a manner similar to as*rpaR*; antisense RNAs have been identified for *Pseudomonas syringae psyR* (29). They are believed to be read-through products from the adjacent *psyI* gene, which is convergently transcribed from and overlaps 69 bp of the 3′-end of *psyR*. One wonders whether this reflects an asRNA regulatory mechanism.

Materials and Methods

Bacteria Strains, Strain Construction, and Growth Conditions. *Escherichia coli* S17-1 (30) and *P. aeruginosa* PA0-T7 (31) were grown in Luria–Bertani broth with aeration at 37 °C. *R. palustris* CGA009 (32) and its derivatives were grown photoheterotrophically at 30 °C in photosynthetic medium (PM) with 10 mM succinate (33) and *p*-coumarate or *p*C-HSL as indicated. Antibiotics were used at the following concentrations: for *E. coli*, 100 μg/mL ampicillin, 25 μg/mL kanamycin, and 20 μg/mL gentamicin; for *P. aeruginosa*, 300 μg/mL carbenicillin and 50 μg/mL gentamicin; and for *R. palustris*, 100 μg/mL gentamicin and 100 μg/mL kanamycin.

We used a variety of *R. palustris* CGA009 mutants including CGA814, which has a *rpaI::lacZ* mutation, and CGA850, a *rpaR* deletion mutant (1, 2). Other mutants were constructed as described below. The as*rpaR* mutant CGA851 was created by inserting an Ω stem-loop terminator fragment (16) between the as*rpaR* transcript start site and the RpaR box (Fig. 2*B*) without disruption of the *rpaR* gene. We used a knockout plasmid to make this mutant. The DNA fragment used to construct the knockout plasmid was created by PCR-sequence overlap extension (SOEing) (34) and corresponded to *R. palustris* genome coordinates 349794–348951. We inserted the 25-bp Ω stem-loop terminator fragment plus a repeat of the last three codons of *rpaR* (TGCTTGTGA) at coordinate 349350 to minimize the effect of the Ω terminator in RpaR activation of *rpaI* transcription. This fragment was cloned into the suicide plasmid pJQ200KS (35) and mated into *R. palustris* CGA009 by conjugation with *E. coli* S17-1 (30) to create strain CGA851.

To engineer *R. palustris* strains that encode a VSVG-tagged RpaR polypeptide, we replaced the native *rpaR* gene with a VSVG-tagged *rpaR* gene. By using SOEing, a 1,616-bp DNA fragment (coordinates 350481–348866) including the VSVG epitope (YTDIEMNRLGK) inserted between the first and second codons of *rpaR* was cloned into pJQ200KS and transferred into either wild-type *R. palustris* to create strain CGA852 or the as*rpaR*-Ω mutant CGA851 to create strain CGA853.

We created the *R. palustris* as*rpaR*-STOP mutant strain CGA854 by replacing the glutamate codon at position 11 in the potential ORF with a stop codon by PCR amplification of a 1,531-bp DNA fragment containing as*rpaR* and flanking sequence (coordinates 350481–348951), cloning this fragment into pJQ200KS, using QuikChange Site-Directed Mutagenesis (Stratagene) to make the mutation, and mating into *R. palustris* CGA009.

Plasmids. The as*rpaR*-expression vector for use in *P. aeruginosa*, pHH100, was constructed by PCR amplification of a 721-bp DNA fragment from coordinates 349349–350069, and cloning in pQF5016b (36). The N-terminal hexaHis-tagged RpaR expression plasmid, pHH101, was constructed by SOEing to yield a DNA fragment containing the entire *rpaR* ORF (coordinates 350081–349350) with six histidine codons inserted between the first and second codons, and cloning into pBBR1MCS5 (37). The as*rpaR-lacZ* construct with an intact RpaR box (pHH102) was created by cloning a 110-bp PCR product containing the as*rpaR* promoter (coordinates 349311–349421) into pBBR1MCS5-lacZ (1). The as*rpaR-lacZ* reporter construct lacking the RpaR box (pHH103) was created in a similar fashion except that the PCR product was shorter (73 bp) because it lacked the RpaR-binding site (coordinates 349348–349421). An as*rpaR*-expression vector for use in *R. palustris*, pHH104, was constructed by using PCR amplification to generate a 722-bp DNA fragment from coordinates 349350–350071, which was cloned into pBBPgdh (38). pHH105 is identical to pHH104, except we mutated the nucleotide at coordinate 349467 from a G to a T by using a QuikChange Site-Directed Mutagenesis kit (Agilent Technologies). This base change modified the 11th codon of the potential ORF identified in the as*rpaR* from a glutamate to a stop codon but did not alter the sequence of RpaR. To generate the anti-as*rpaR* expression vector, pHH106, we PCR amplified a 204-bp DNA fragment that encodes an interior portion of *rpaR* sense mRNA (coordinates 349592–349389) and cloned it into pBBPgdh.

Northern Blotting. We isolated total RNA from *R. palustris* cultures at an optical density at 660 nm of 0.45 as described (15, 39). RNA (10 μg) was denatured, separated by agarose-gel electrophoresis [1.2% (wt/vol) agarose], and transferred to a positively charged nylon membrane by using a NorthernMax-Gly Gel Prep kit (Ambion). Membranes were probed with either a 207-bp, strand-specific, DIG-labeled RNA probe specific to *rpaR* sense or to as*rpaR* RNA (coordinates 349386–349592). The RNA probes were created by transcription-labeling and hybridization as described by the manufacturer (DIG Northern Starter kit; Roche Applied Science).

Primer Extension Analysis. We annealed 20 μg of total RNA from log-phase *R. palustris* cultures with 10 nM of a 6-FAM–labeled primer complementary to the terminal 118 bp of the *rpaR* ORF. Reverse transcription was with SuperScript III reverse transcriptase (Life Technologies), and the cDNA was purified for GeneScan sequencing analysis as described (2).

Analysis with 3′-RACE. We used 10 μg of total RNA isolated from log-phase *R. palustris* grown in the presence of 250 nM *p*C-HSL and attached a poly-A linker to the 3′-terminal of RNA using *E. coli* Poly(A) polymerase (New England Biolabs). After cleanup using the RNAeasy kit (Qiagen), 25% of the reaction volume was further processed by using the 3′-RACE System for Rapid Amplification of cDNA Ends as described by the manufacturer (Life Technologies). Two rounds of 3′-RACE were performed by using a distinct primer unique to as*rpaR* each time (round 1 unique primer corresponded to genome coordinate 349662–349680; round 2–349469-349489). Eleven 3′-RACE products were TA-cloned into pCR2.1 TOPO (Life Technologies) and sequenced (Table S1).

Promoter Activity Assays. To monitor the *rpaI* promoter levels, we used either *R. palustris* CGA814, which harbors a chromosomal *rpaI-lacZ* fusion, or the plasmid pPrpaI (1), and to monitor as*rpaR* promoter activity, we used *lacZ* transcriptional fusion plasmids (pHH102 and pHH103). β-Galactosidase levels were measured with a Tropix Galacto-Light Plus kit (Applied Biosystems).

Measurement of *p*C-HSL. To measure *p*C-HSL levels in *R. palustris* cultures, bacteria were grown with 0.5 mM *p*-coumarate (starting OD_{660} 0.04) for the indicated times. *p*C-HSL was extracted from cultures with acidified ethyl acetate (0.1 mL of glacial acetic acid per liter) and measured by using a described *p*C-HSL bioassay (1).

Western Blotting. To detect VSVG-RpaR from *R. palustris* and hexaHis-RpaR from *P. aeruginosa*, cells were grown to late-log phase, harvested by centrifugation, suspended in SDS/PAGE buffer, and lysed by boiling and sonication. Cell lysates (7.5 μg of protein per lane for *P. aeruginosa*; 18 μg per lane for *R. palustris*) were separated on duplicate 12% acrylamide Tris-glycine SDS/PAGE gels. One gel was stained with Coomassie brilliant blue stain, and the other was electroblotted onto a PVDF membrane (Millipore). VSVG-tagged RpaR was detected with VSVG antiserum (Sigma Chemical), which was detected with secondary anti-rabbit horseradish peroxidase-conjugated IgG and chemiluminescent substrate (Pierce Protein Research). HexaHis-RpaR was visualized by using a SuperSignal West HisProbe kit (Pierce Protein Research).

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Supporting Information

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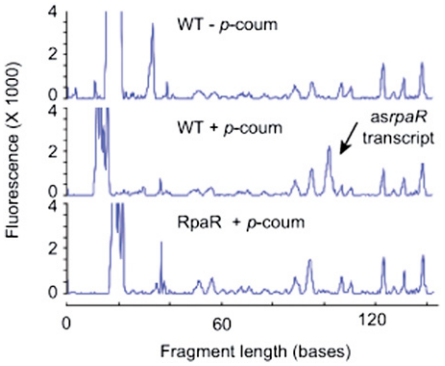

Fig. S1. S1 nuclease protection analysis of the as*rpaR* transcript from the *R. palustris* wild-type (*Top* and *Middle*) and the RpaR-mutant (*Bottom*) strains in the absence (*Top*) or presence (*Middle* and *Bottom*) of 250 nM *p*-coumarate. The as*rpaR* product (indicated by arrow) was only detected in the wild type grown with *p*-coumarate. The as*rpaR* fragment was 99 bp in length, thus mapping to a guanine 41 bp from the 3′ end of *rpaR* (Fig. 2*B*). The 324-bp probe for the assay was generated by PCR amplification with genomic DNA as template, a 6-FAM–labeled forward primer (CCTGGGAAATCTCGGTAATCC) and unlabeled forward primer (GCGCTCGGCGACGTAGATCT). S1 nuclease protection assay protocol was as described (1).

1. Hirakawa H, et al. (2011) Activity of the Rhodopseudomonas palustris p-coumaroyl-homoserine lactone-responsive transcription factor RpaR. *J Bacteriol* 193:2598–2607.

Table S1. Summary of detected as*rpaR* 3′-RACE products

Product end*	Length†
349695	305
349740	350
349771	381
349776	386
349784	394
349792	402
349797	407
349807	417
349829	439
349852	460
349858	468

*Genome coordinate of the product end.
†Length of the as*rpaR* product, assuming a start site at genome coordinate 349391.